Manual RNA extraction from biological samples

This protocol is a copy of the standard operating procedure used by the OIE/FAO International Reference laboratory for AI at the Animal and Plant Health Agency. If you have any technical queries please contact AIWRL@apha.gov.uk

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1. INTRODUCTION

1.1 Purpose/Scope of this Protocol

1.1.1 To extract RNA from biological samples using commercial extraction kits for subsequent genetic analysis techniques.

1.2 Background information

1.2.1 RNA extraction kits are designed to isolate total RNA from small quantities of starting material.

1.2.2 They are a fast and simple method for the extraction of RNA from a range of samples such as clarified tissue homogenate supernatants, egg amniotic/allantoic fluid and tissue culture cells/supernatant.

1.2.3 Once extracted the purified RNA can be used in a range of genetic analysis techniques.

1.2.4 Samples are lysed under highly denaturing conditions to inactivate Rnases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the silica-gel based membrane of a mini spin column and contaminants are washed away. RNA is eluted in an Rnase-free buffer or water by the use of microcentrifugation.

2. SAFETY

2.1 It is the responsibility of the individual laboratory to ensure all work described in this protocol is conducted to a high safety standard. This includes an awareness of risks relating to *e.g.* dangerous or toxic chemicals, potentially hazardous procedures etc. Here local safety rules in your laboratory should be understood by all relevant members of staff.

2.2 ⚠ Areas within this procedure which refer to Safety Critical activities are denoted in the paragraph number column with the sign to highlight these areas to users.

2.3 Due to the nature of samples submitted for avian or mammalian virological investigation clinical material must be treated as potentially infected with influenza A virus or other zoonotic pathogens that might affect human beings such as AOAV-1. All manipulations prior to inactivation of material in AVL lysis buffer and 100% ethanol must be carried out in a class 1, 2 or 3 microbiological safety cabinet (MSC) according to the BAAC RA 2006/06 and Containment Laboratories Microbiological Safety Cabinet Class Assignment Table.

Please note: The minimum time required in order for virus to be inactivated in lysis buffer AVL is **10 minutes.** After the addition of 100% ethanol and surface decontamination following local procedures, it is safe to remove the samples from the MSC to a lower containment level.

2.4 AVL is chaotropic and will dissolve proteins. Therefore, gloves must be worn at all times and care must be taken not to get this material on skin or in eyes. In this event rinse the affected area **IMMEDIATELY** with tap water and consult with onsite medical staff if burns have occurred.

2.5 When decontaminating items for removal from an MSC the approved disinfectant – 1% Virkon S with a contact time of 10 minutes – must be used.

3. MATERIALS

3.1 Documentation and software

QIAamp viral RNA mini kit handbook

Other procedures referred to in this procedure:

(a) Preparation of clinical samples

(b) Extraction of nucleic acids from swabs using the QiAGEN BioRobot Universal

3.2 Chemicals and reagents

3.2.1 All reagents are supplied with the kits except the following:

3.2.2 96-100% ethanol

3.2.3 Brain heart infusion broth (BHIB) + antibiotics (1000IU penicillinG;10μg/ml amphotericin B; 1mg/ml gentamicin)

3.2.4 Qiagen QIAamp viral RNA mini kit – Part number 52904 (50 reaction) or 52906 (250 Reactions).

⚠AVL Lysis buffer
Carrier RNA
AW1: Wash buffer 1
AW2: Wash buffer 2

3.3 Equipment

3.3.1

Microcentrifuge tubes (1.5ml)	Disposable gloves
Sterile, Rnase-free pipette tips with aerosol barrier	Pipettes
Microcentrifuge (with rotor for 2ml tubes)	An appropriate class of microbiological safety cabinet (MSC).
Vortex mixer	
-70°C freezer or below	

4. PROCEDURE/METHOD

4.1 Test Reliability

4.1.1 An annual proficiency panel is distributed to European and third party national avian influenza laboratories. Results from all laboratories are discussed at meetings and a report published which may include recommendations.

A positive extraction control must be included for manual extractions of clinical material. However, a positive control is not required when extracting egg amplified material. If samples from a proficiency panel are being extracted these are treated as clinical material and a positive control MUST be included. Negative RNA extraction controls are included and examined for every run and the presence of any contamination is recorded.

The manual extraction kits are used regularly for extraction of known positive research material, and in conjunction with other confirmatory tests. The first extraction carried out using a new kit is recorded as evidence of fitness for purpose.

The integrity and quality of each nasal swab submitted for testing **must be inspected** before processing. If a swab is excessively soiled with material, then clarification of swab suitability will be required from the test consultant.

4.2 Swab Processing

4.2.1 ⚠ Addition of sample to AVL Lysis buffer and subsequent addition of ETOH, must be carried out in an appropriate class of MSC according to the BAAC RA 2006/06 and local Containment Laboratories Microbiological Safety Cabinet Class Assignment Tables

4.2.2 BHIB is dispensed in 1ml aliquots into 1.5ml Eppendorf tubes and stored at +4^{o}C prior to use.

4.2.3 Label tubes containing BHIB with swab identification.

4.2.4 Put the cotton wool end of swab into the appropriately labelled tube containing BHIB. Using a pair of single use scissors or bone cutters snip the shaft of the swab near to the fibre end. Close the lid of the tube.

4.2.5 ⚠ Immediately place the remainder of the swab in a sharps bin and dispose of the plastic sheath.

4.2.6 Dip the end of the bone crushers or scissors into a beaker of 100% ethanol or use a new pair of scissors as appropriate to the samples being processed.

4.2.7 Repeat this procedure for all swabs.

4.2.8 Allow to stand for a minimum of 2 minutes to allow solid material to settle.

4.2.9 The swab eluent can now be used for RNA extraction, (step 4.3.6 above).

4.3 QIAamp viral RNA mini kit (Centrifugation kit)

4.3.1 Equilibrate all buffers and samples to room temperature before use.

4.3.2 This kit can be used on plasma, serum, cell free body fluids, swab and cell culture supernatants and tissue homogenates

4.3.3 Check AVL buffer for precipitate, and if necessary incubate at 80°C until the precipitate is dissolved. Add 1ml of AVL buffer to one tube of lyophilised carrier RNA. Dissolve RNA thoroughly. Transfer to the AVL bottle, and mix thoroughly before using AVL buffer. Once the carrier RNA has been resuspended the solution is stored at +2-8°C and is stable for 48 hours - the preparation date and an expiry date MUST be clearly written on the bottle. Alternatively aliquot any remaining AVL/carrier RNA in 560µl volumes and freeze at -20°C.
DO NOT warm Buffer AVL–carrier RNA solution more than 6 times

(2). DO NOT incubate at 80°C for more than 5 min. Frequent warming and extended incubation will cause degradation of the carrier RNA, leading to reduced recovery of viral RNA and eventually false negative RT-PCR results, particularly when low-titer samples are used.

Carrier RNA is routinely added to AVL buffer before use, however some applications require the omission of carrier RNA to the AVL, where its presence may interfere with downstream testing, for example when the resulting RNA is likely to be submitted for whole genome testing.

4.3.4 AW1 and AW2 buffer requires the addition of ethanol (96-100%), volume required is printed on each of the bottles.

This buffer is stable for 1 year when stored closed at room temperature (to lie within the range 15-25$_o$C) but only until kit expiration date.

4.3.5 ⚠ Prepare the required number of Eppendorfs containing 560µl AVL/carrier RNA buffer, including the number of additional volumes required for positive and negative controls.

4.3.6 ⚠ In an appropriate class of MSC, add 140µl of specimen to 560µl AVL buffer containing carrier RNA in a microcentrifuge tube. Swab samples are prepared according to Section 4.2. Mix thoroughly.

NB: High titre samples can cause cross contamination and therefore care must be taken in the addition of samples to AVL to avoid this.

Inactivated samples can be added to AVL without the need of a MSC.

4.3.7 Incubate at room temperature for 10 minutes.

4.3.8 ⚠ Add 560µl ethanol to the sample, mix by gentle aspiration for 15 seconds.

4.3.9 At this point the reaction can be removed from the MSC according to local decontamination procedures.

4.3.10 Briefly centrifuge the tube to remove drops from the inside of the lid.

The following centrifugation steps are carried out at room temperature and at approximately 6000g or full speed

4.3.11 Add 630µl of the solution to a spin column in a 2ml collection tube and centrifuge 6000 x g (8000rpm) for 1 minute.

4.3.12 Place spin column in a clean collection tube and discard the tube

containing the filtrate.

4.3.13 Repeat steps 4.3.11 and 4.3.12 until the full sample volume has been loaded.

4.3.14 Add 500µl AW1 buffer to spin column and centrifuge 6000 x g (8000rpm) for 1 minute.

4.3.15 Repeat step 4.3.12.

4.3.16 Add 500µl AW2 buffer to spin column and centrifuge 20,000 x g (13,000rpm) for 3 minutes.

4.3.17 **Recommended**; It can be beneficial to downstream processes to performa further centrifugation of the samples to remove any residual buffer. Note the kit does not contain enough collection tubes for this additionalstep! Place spin column in a **clean 1.5ml microcentrifuge tube (with the lid cut off)** and discard the tube containing the filtrate. Centrifuge atfull speed for a minute.

4.3.18 Place spin column in a clean 1.5ml microcentrifuge tube and discard the tube containing the filtrate.

4.3.19 Add 50µl of room temperature RNase free water. Incubate at room temperature for 1 minute. Centrifuge at 6000g for 1 minute.

4.3.20 Discard spin column and transfer RNA to a suitably labelled 1.5ml tube.

4.3.21 The RNA is now ready for genetic testing or storage at -70^{o}C until required.